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Acute exercise modulates *Trim63* and *Bmal1* in the skeletal muscle of IL-10 knockout mice

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ABSTRACT

The anti-inflammatory role of physical exercise is mediated by interleukin 10 (IL-10), and their release is possibly upregulated in response to IL-6. Previous studies demonstrated that mice lacking IL-6 (IL-6 KO mice) exhibited diminished exercise tolerance, and reduced strength. Rev-erbα, a transcriptional suppressor involved in circadian rhythm, has been discovered to inhibit the expression of genes linked to bodily functions, encompassing inflammation and metabolism. It also plays a significant role in skeletal muscle and exercise performance capacity. Given the potential association between Rev-erbα and the immune system and the fact that both pathways are modulated following acute aerobic exercise, we examined the physical performance of IL-10 KO mice and analyzed the modulation of the atrophy and Rev-erbα pathways in the muscle of wild type (WT) and IL-10 KO mice following one session of acute exercise. For each phenotype, WT and IL-10 KO were divided into two subgroups (Control and Exercise). The acute exercise session started at 6 m/min, followed by 3 m/min increments every 3 min until animal exhaustion. Two hours after the end of the exercise protocol, the gastrocnemius muscle was removed and prepared for the reverse transcription-quantitative polymerase chain reaction (RT-q-PCR) and immunoblotting technique. In summary, compared to WT, the IL-10 KO animals showed lower body weight and grip strength in the baseline. The IL-10 control group presented a lower protein content of BMAL1. After the exercise protocol, the IL-10 KO group had higher mRNA levels of *Trim63* (atrophy signaling pathway) and lower mRNA levels of *Clock* and *Bmal1* (Rev-erbα signaling pathway). This is the first study showing the relationship between Rev-erbα and atrophy in IL-10 KO mice. Also, we accessed a public database that analyzed the gastrocnemius of MuRF KO mice submitted to two processes of muscle atrophy, a denervation surgery and dexamethasone (Dexa) injections. Independently of knockout, the denervation demonstrated lower *Nr1d1* levels. In conclusion, IL-10 seems to be a determinant in the Rev-erbα pathway and atrophy after acute exercise, with no modulation in the baseline state.

1. Introduction

Interleukin 10 (IL-10) is currently recognized as an anti-inflammatory and immunosuppressive factor, demonstrating a central role in controlling innate and adaptive immune responses and acting in the defense against infections. Furthermore, the reduced expression of IL-10 is associated with several autoimmune disorders [1]. As an anti-inflammatory cytokine, IL-10 suppresses the capacity of monocytes and macrophages, as well as the production of pro-inflammatory cytokines, including IL-6 and tumor necrosis factor-alpha (TNF-α) [2]. Maintaining tissue homeostasis critically depends on pro- and anti-inflammatory cytokines balance [3].

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Skeletal muscle is acknowledged as a "secretory organ" and releases myokines in response to contraction [4]. The immunological alterations of exercise can be detected through a selection of immune cells and pathways [3]. Moderate physical exercise is an anti-inflammatory intervention that improves physical function by reducing the risk of chronic diseases [5]. The duration, intensity, and exercise-induced skeletal muscle trauma can impact the release of anti-inflammatory cytokines [6]. The impact of physical exercise on interleukin-10 (IL-10) concentrations endorses exercise as a therapeutic approach for reducing inflammation [7]. The circulating levels of both IL-10 and IL-1 receptor antagonists (IL-1ra) increase after exercise [8], and their release is possibly upregulated in response to IL-6 [9]. IL-1ra, a receptor antagonist of IL-1 (a pro-inflammatory cytokine), can competitively bind with IL-1 receptor type I (IL-1R1), thus blocking cell activation by the cytokine [10].

Regarding acute physical activity, IL-6 triggers the generation of IL-1ra and IL-10 by blood mononuclear cells, thereby promoting the occurrence of anti-inflammatory cytokines [8,11,12]. While IL-6 is known for its pro-inflammatory role, it also has anti-inflammatory effects during exercise. IL-6 is a crucial ligand of muscle contraction and adjusts hepatic glucose output [13]. The IL-6-Signal transducer and activator of transcription 3 (STAT3) pathway promote muscular adaptations after training, such as mitochondria biogenesis, as well as increased mitochondria activity and exercise tolerance [14]. Once several studies demonstrated that IL-6 knockout (KO) mice had a lower exercise tolerance [15–21], the first aim of the present study was to verify if another anti-inflammatory cytokine, IL-10, can affect the physical performance in this transgenic mouse (IL-10 KO).

Walston *et al.* [22] showed higher serum levels of IL-6 in IL-10 KO mice. The authors also verified lower strength in the IL-10 KO group than in the wild-type control group, suggesting that this transgenic mouse could be a frailty model [22]. Based on these findings, the second aim was to verify if the frailty could be due to the upregulation of the atrophy pathway (*Trim63*, also known as *Murf1*; and *Fbxo32*, also known as *Atrogin1*).

The circadian clock impacts the immune system, and disturbance of circadian rhythms has been connected to inflammatory pathologies [23]. The core machinery of the circadian clock involves autoregulatory transcription and translation feedback loops. The primary arm of the core loop comprises Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like 1 (BMAL1). The secondary arm of the loop incorporates the retinoic acid-related orphan receptors (RORα/β/γ, NR1F1/2/3) and REV-ERBα/β (NR1D1/2), which exercise contrary effects on the molecular clock by activating or repressing BMAL1 transcription [24]. The Rev-erbα is a transcriptional silencer that suppresses the expression of genes related to physiological roles, such as inflammation and metabolism [25]. Wolff *et al.* [26] observed pro-inflammatory cytokine expression attenuation in response to using a Rev-erbα agonist (SR9011) in microglia, whereas the anti-inflammatory cytokine IL-10 expression was stimulated. However, it is unknown the effects of IL-10 on Rev-erbα. Also, it was demonstrated that the nuclear receptor of subfamily 1 group D member 1 (*Nr1d1* - the gene encoding the Rev-erbα protein) plays an important role in skeletal muscle and exercise performance capacity [27]. Rev-erbα global knockout mice had decreased performance, lean muscle mass, reduced cross-sectional area of the fibers, and increased expression of atrophy-related genes (*Murf1* and *Atrogin-1*) in the skeletal muscle [27,28].

Since Rev-erbα seems to relate to the immune system, and both pathways are modulated after an acute aerobic exercise session, the third aim of this study was to verify the effects of the global deletion of IL-10 on the mRNA levels and protein content of the Rev-erbα pathway in the skeletal muscle of sedentary and exercised mice.

The present study will help to comprehend the mechanism by which IL-10 influences muscle physiology, particularly in the context of a stimulus like physical exercise and its correlation with a pivotal protein in the circadian rhythm, to facilitate future investigations into the interplay between these molecules in the context of muscle mass depletion and fragility, both prevalent in the elderly population affected by sarcopenia.

2. Methods

2.1. Experimental animal

Male C57BL/6 mice with two to three months, from the Central Animal Facility of the Ribeirão Preto campus from the University of São Paulo (USP) were used for the wild-type (WT) group. IL10$^{-/-}$ mice from the Jackson Laboratory (https://www.jax.org/strain/002251) were bred and used as the IL-10 knockout group (IL-10 KO), with the same age and gender as the WT group. Mice were housed in sterile microisolators (two to three animals per cage) in a ventilated rack (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) with controlled temperature (22 ± 2 °C) in a light–dark inversion cycle from 12:12 h (light: 6:00 pm to 6:00 am, dark: 6:00 am to 6:00 pm), with food (Purina chow) and water *ad libitum*. All experimental procedures followed the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee of the University of São Paulo (2021.1.43.90.0). Before any experimental procedure, 10 wt mice and 10 IL-10 KO mice were weighed. After, randomly, five to six animals for WT (n = 5–6) and IL-10 KO (n = 5–6) were submitted to the incremental load, grip strength, and rotarod tests to verify how the genetic modification interfered with the performance tests. After three days of testing, WT and IL-10 KO mice were distributed into two groups with two subgroups for each phenotype: WT Control (sedentary; Basal, n = 5), IL-10 KO (sedentary; Basal, n = 5), WTEx (submitted to aerobic exercise, n = 5), and IL-10 KO-Ex (submitted to aerobic exercise, n = 6). The exercised groups were submitted to aerobic exercise. Fig. 1 summarizes the experimental design.

2.2. Incremental load test

The animals were acclimated in the active phase to performing physical activity on a treadmill (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) for 5 days, 10 min per day at a 6 m/min velocity. The progressive test began at an initial velocity of 6 m/min, with no inclination, and increased by 3 m/min every 3 min until the mice reached exhaustion, which was determined when they touched the end of the treadmill 5 times within a 1-minute timeframe [29].

2.3. Grip strength test

To perform the grip strength assessment, the mice were grasped by the tail, enabling them to grip the horizontally placed metal bar of the grip strength device using all four paws (Grip Force, Avs Projetos®, São Carlos, São Paulo, Brazil). The mice underwent five consecutive tests to measure their force. The mean of the recorded values registered on the metal bar was considered a performance parameter [29].

2.4. Rotarod test

The rotarod apparatus was set to initiate movement at a speed of 1 rpm and gradually accelerate until it reached 40 rpm after 300 s. The acceleration remained consistent throughout the entire test. Each animal was given three chances, with a 10-second interval between attempts, and the time (in seconds), until each animal fell was documented [29].

2.5. Acute aerobic exercise

One week after the tests, the exercise group was submitted to the incremental test, as an acute aerobic exercise protocol, just one session for one day, during the mice's active phase. The exercise protocol consisted of one acute session on the treadmill, starting at 6 m/min,

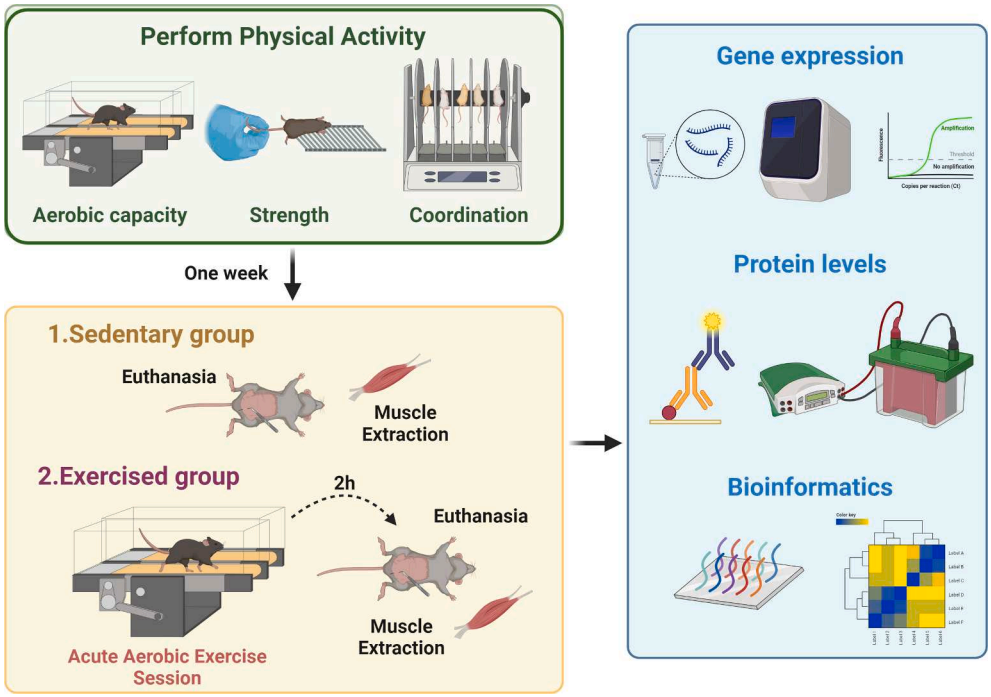

Fig. 1. Experimental design. The WT and IL-10 KO mice underwent incremental load, grip strength, and rotarod tests. The exercised groups were submitted to an acute aerobic exercise session. WT Control (sedentary; Basal), IL-10 KO (sedentary; Basal), WTEx (submitted to aerobic exercise), and IL-10 KO-Ex (submitted to aerobic exercise). Figure by Biorender® (agreement number: OM269SEOTQ).

followed by increments of 3 m/min every 3 min until the animal was exhausted. Voluntary exhaustion of the mice occurred when the mice touched the end of the treadmill 5 times within a 1-minute interval. The time of exhaustion of each animal was different, however, it varied from 10 to 17 min.

2.6. Euthanasia and gastrocnemius muscle extraction

Mice were anesthetized with an intraperitoneal injection of xylazine (10 mg/kg body weight) and ketamine (100 mg/kg body weight) at baseline (no exercise) and 2 h after the exercise protocol. As soon as the loss of pedal reflexes confirmed the effect of anesthesia, the gastrocnemius muscle was removed and prepared for a polymerase chain reaction – quantitative reverse transcriptase (RTq-PCR) and immunoblotting technique.

2.7. Transcription-quantitative polymerase chain reaction (RT-q-PCR)

The RT-q-PCR technique was performed as previously described [30,31]. Quantitative real-time PCR was performed to analyze the relative mRNA expression of nuclear receptor subfamily 1 group D member 1 (*Nr1d1*), Atrogin-1 (*Fbxo32*), E3 ubiquitin-protein TRIM63 ligase (*Trim63* or *Murf1*), brain and muscle ARNT as protein 1 (*Bmal1* or *Arntl1*) and kaput circadian locomotor output cycle (*Clock*) (Table 1). *Gapdh* was used as a reference gene to normalize the data. Relative quantitation was calculated by the 2 -ΔΔCT method using the Thermo Fisher Cloud Software, version RQ 3.7 (Life Technologies Corporation, Carlsbad, CA, USA).

Table 1
Primer sequences.

Gene	Forward (5′-3′)	Reverse (5′-3′)
Bmal1	GGACTTCGCCTCTACCTGTTC	ACCCGTATTTCCCCGTTC
Clock	ATGGTTTACCGTAAGCTGTAG	CTCGCGTTACCAGGAAGCAT
Fbxo32	CAAAGGAAGTACGAAGGAGAGCG	TCAGCTCCAACAGCCTTACTA
Nr1d1	GGTGTTTGGCGCAGCACCTT	CTCTGGGATAAATGCCCGAAGCT
Trim63	CAGGCTGCGAATCCCTACTG	GCCGGTCCATGATCACTTCA
Gapdh	AAGAGGGATGCTGCCCTTAC	CGGGACGAGGAAACACTCTC

2.8. Immunoblotting technique

The immunoblotting technique was performed as previously described [30,31]. Antibodies were used: Glycealdehyde-3-phosphate dehydrogenase (GAPDH; #2118), interleukin 6 (IL-6;#12153), tumor necrosis factor-alpha (TNF-α; #3707) and BMAL1 (#14020) from Cell Signaling Technology (Cell Signaling Technology, MA, USA); nuclear receptor subfamily 1 group D member 1 (Rev-erbα, SC-393215) and E3 ubiquitin-protein ligase TRIM63 (MuRF1; SC-398608) from Santa Cruz Biotechnology (Dallas, Tx, USA); Atrogin-1 (Atrogin; ab-168372) from ABCAM (Cambridge, UK). Primary antibodies were used at a dilution of 1:1000, and secondary antibodies were used between 1:10,000 and 1:20,000. Images were acquired by ChemiDocTM (Bio-Rad Laboratories, California, USA) and quantified using Image Lab software. Figures were checked for data integrity using the Proofig pipeline (https://www.proofig.com).

2.9. Publicly available transcriptomic datasets of mice

We accessed a public database of mice samples with and without exercise in the gastrocnemius and soleus to verify the behavior of the genes studied in the preset manuscript in different types of skeletal muscle. The mice dataset in Fig. 5A and 5B were obtained from the GEO Series accession number GSE198266 [32]. The mice dataset in Fig. 5C and 5D were obtained from the GEO Series accession number GSE179163. Heatmaps were built using Morpheus (https://software.broadinstitute.org/morpheus/).

2.10. Statistics

Results were expressed as mean ± standard deviation (SD). The Shapiro-Wilk W test verified data normality. For the physical tests, as it constituted a comparison only between WT and IL-10 KO, a Student's *t*-

test was used. When normality was confirmed, a two-way analysis of variance (two-way ANOVA) was used to compare a specific gene/protein expression response between activity (sedentary *versus* exercise) and groups (WT *versus* IL-10 KO). Bonferroni's post hoc test was performed when two-way ANOVA indicated significance. All statistical analyses were set at $p < 0.05$ and bilateral. Statistical analyses were performed using GraphPad Prism v.8.0.1 for Windows.

3. Results

3.1. IL-10 KO mice had lower body weight and strength

Body weight (Fig. 2A) and grip strength test (Fig. 2C) were lower for the IL-10 KO group when compared to the WT group. Regarding the incremental test (Fig. 2B) and the rotarod test (Fig. 2D), there was no difference between the groups.

3.2. Acute exercise increased Trim63 levels in the IL-10 KO mice

Il-10 mRNA levels were lower for the WTEx group when compared to the WT group (Fig. 3A). The mRNA levels of *Fbxo32* (Fig. 3C) and *Nr1d1* (Fig. 3D) did not differ between the experimental groups. Regarding the mRNA levels of *Trim63* (Fig. 3B), the IL-10 KO-Ex group presented higher mRNA levels than the other groups. *Clock* (Fig. 3E) and *Bmal1* (Fig. 3F) mRNA levels were higher for the WTEx group than the IL-10 KO-Ex group.

3.3. IL-10 KO mice had a lower protein content of BMAL1

Fig. 4A, 4B, and 4C show the membranes of the immunoblotting experiment. Regarding MuRF-1 (Fig. 4D), Atrogin-1 (Fig. 4E), Rev-erbα (Fig. 4F), IL-6 (Fig. 4H), and TNFα (Fig. 4I), no difference between the experimental groups was verified. The BMAL1 protein content (Fig. 4E) was lower for the IL-10 group than for the WT group.

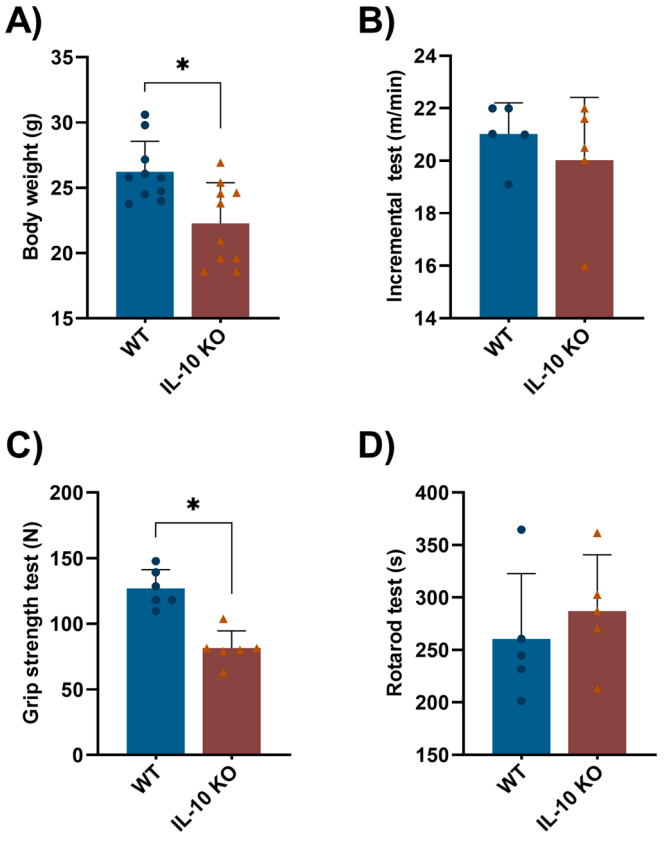

Fig. 2. Body weight and performance Test. Body weight (A), Incremental Test (B), Grip Strength Test (C), and Rotarod Test (D). The data correspond to the mean ± SD of n = 10 for body weight and n = 5 to 6 mice/group for the other parameters. *$p \leq 0.05$. WT.

3.4. The Nr1d1 was modulated in the gastrocnemius and soleus of mice in different conditions

Initially, we conducted an analysis using publicly available data to investigate whether the expression of specific muscle fiber types would have an impact on the regulation of gene responses within the pathways under investigation in our current study. According to Fig. 5A, the genes modulated in the gastrocnemius from control to exercise were: *Fbxo32, Trim63, Arntl, Arnt2, Nr1d1, Tnf, and Il-6*. For the soleus (Fig. 5B), the *Fbxo32, Trim63, Arntl, Arnt2*, and *Nr1d1* had the same behavior. The *Arnt*, *Clock*, *Il6ra*, and *Il10ra* were also modulated for the soleus.

Subsequently, we explored the utilization of well-established models to induce muscle mass loss and subsequently frailty, such as denervation and treatment with dexamethasone, to compare with *Murf1* knockout animals, a critical gene within the atrophy pathway. Also, our data showed an increase in the mRNA levels of *Trim63* (*Murf1*) in the IL-10 KO-Ex group. Our objective was to elucidate the potential relationship between IL-10 and Rev-erb pathway genes under these conditions, as IL-10 knockout animals have been previously identified as a frailty model by Walston *et al.* [22].

In Fig. 5C, it is possible to verify that the denervation, independently of knockout, demonstrated lower *Nr1d1*. The MuRF KO denervated group had modulation of *Fbxo32*, another atrophy gene, and *Il10* and *Il6* receptors. In Fig. 5D, the dexamethasone injection modulated the *Trim63* and *Fbxo32* for the WT group. The *Il10* and *Il6* receptors responded differently than the denervated animals. Just the WT with Dexa had a modulation on the *Nr1d1* levels.

4. Discussion

In the present investigation, we verified the physical performance in IL-10 KO mice, as well as the modulation of the atrophy and Rev-erbα pathways in the muscle of WT and IL-10 KO mice after an acute exercise session. We also studied the current genes in two public databases as a secondary analysis. In summary, IL-10 KO animals had lower body weight and grip strength. The IL-10 control group presented lower protein content of BMAL1 and, after the exercise protocol, had higher *Trim63* (atrophy signaling pathway) but lower *Clock* and *Bmal1* mRNA levels (Rev-erbα signaling pathway). Public database assessment showed that the gene *Nr1d1* was modulated in the gastrocnemius and soleus of mice in different conditions.

Muscle fragility is multisystem and may be due to genetic alterations related to age and the state of chronic diseases [33]. According to the data obtained in the present study, Walston *et al.* (2008) also verified that the IL-10 KO group had an earlier decline in muscle strength than the control group [22]. The authors also demonstrated that strength declined is an estimated time–genotype interaction [22]. Although these are preliminary results, the decline in the strength test provides evidence for using the IL-10 KO animal model to study human fragility.

The WTEx group presented lower *Il-10* mRNA levels than its control group but without changes in the protein levels of inflammatory cytokines between the experimental groups. Several studies show that after a prolonged acute exercise session, there is a significant increase in serum inflammatory cytokines (TNF-α and IL-6) and anti-inflammatory cytokines (IL-10) [34–36]. It is also well established in the literature that cytokines have a short half-life in circulation and that IL-10 levels peak at the end of exercise or immediately after [7,37,38]. After an acute exercise (5 continuous km on a treadmill at 70 % of maximum oxygen uptake – VO2), the maximum serum concentrations of IL-10 reached 60 min at the end of the acute exercise compared to baseline levels [39]. Louis *et al.* [40] analyzed the time course of *Il-6* and *Tnf-α* in the

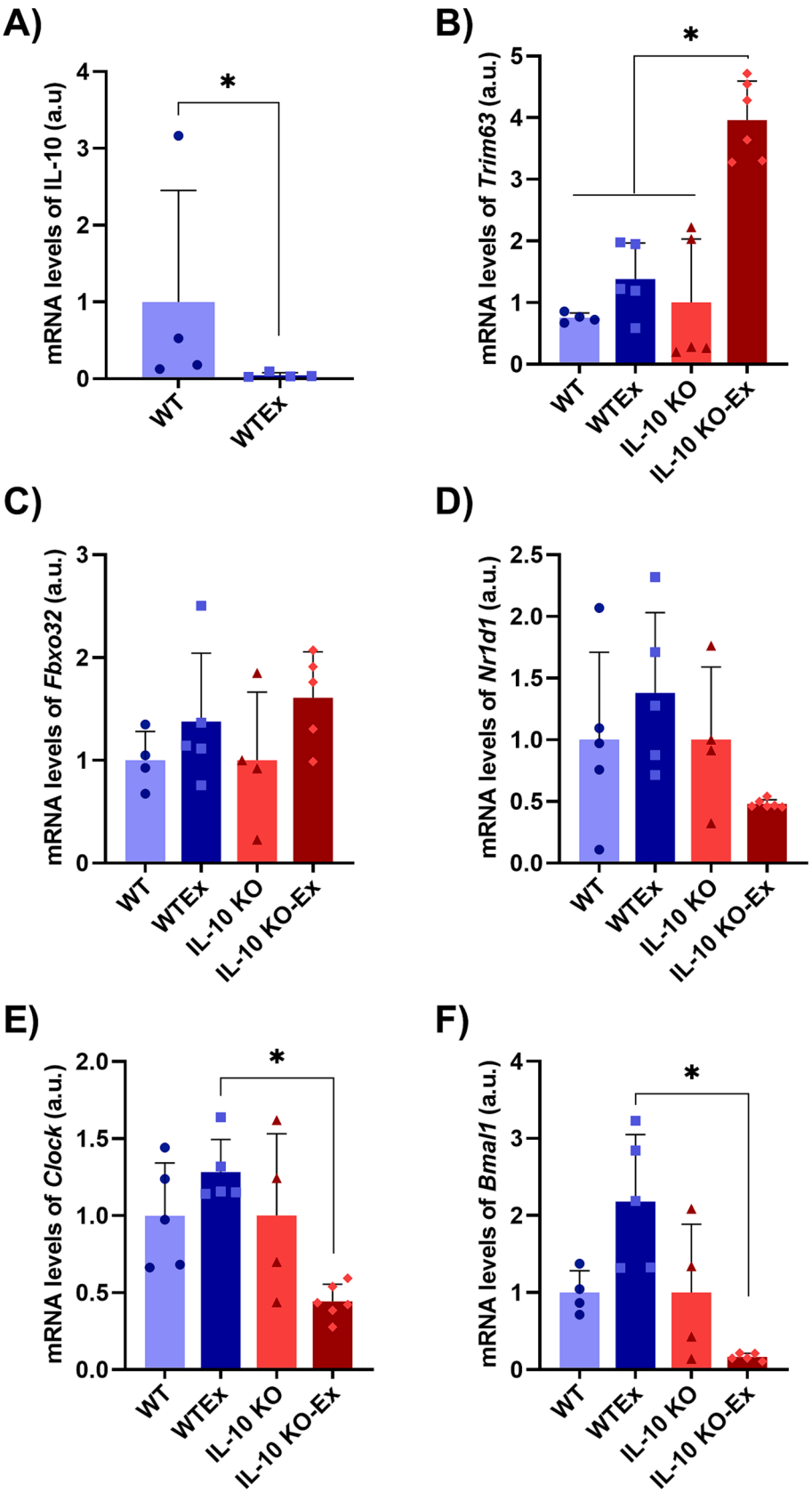

Fig. 3. Gastrocnemius gene levels. Gastrocnemius mRNA levels of (A) *IL-10*, (B) *Trim63*, (C) *Fbxo32,* (D) *Nr1d1*, (E) *Clock,* and (F) *Bmal1*. The data correspond to the mean ± SD of n = 4 to 6 mice/group *p ≤ 0.05. Control (WT or IL-10 KO; sedentary; Basal), Exercised (WTEx or IL-10 KO-Ex; submitted to aerobic exercise). a.u: arbitrary units.

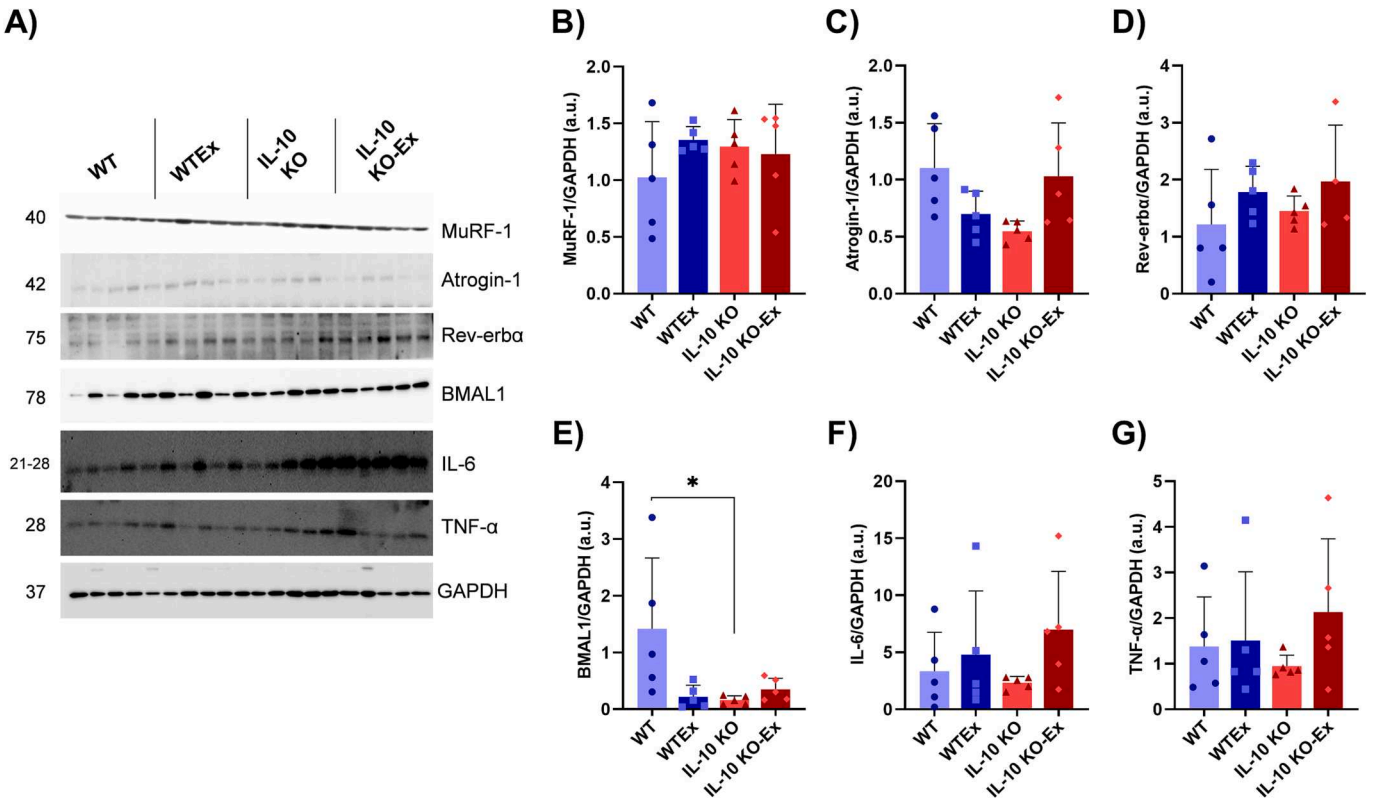

Fig. 4. Gastrocnemius protein content. (A) Representative membranes. The gastrocnemius protein content of (B) MuRF-1, (C) Atrogin-1, (D) Rev-erbα, (E) BMAL1, (F) IL-6, and (G) TNFα. GAPDH normalized all proteins. The data corresponds to the mean ± SD of n = 5 mice/group, except for Rev-erbα, which n = 4 for IL-10 KO-Ex. *p ≤ 0.05. Control (WT or IL-10 KO; sedentary; Basal), Exercised (WTEx or IL-10 KO-Ex; submitted to aerobic exercise). a.u: arbitrary units. The representative membranes were checked for data integrity using the Proofig pipeline (https://www.proofig.com).

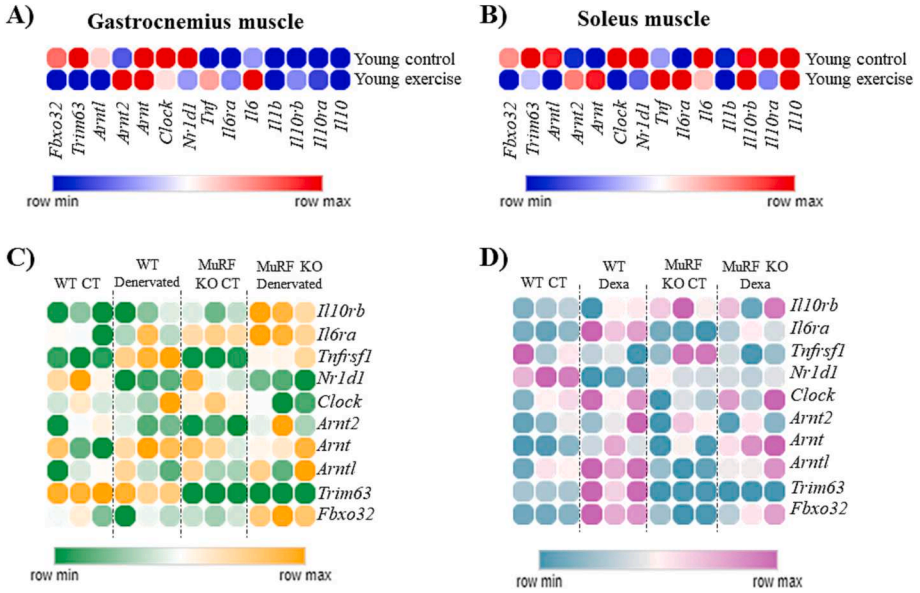

Fig. 5. Heatmap datasets. A) Heatmap of atrophy and Rev-erbα pathways in the gastrocnemius of young control and exercise mice (n = pool of 3). B) Heatmap of atrophy and Rev-erbα pathways in the soleus of young control and exercise mice (n = pool of 3). C) Heatmap of atrophy and Rev-erbα pathways in the gastrocnemius of Wild-type (WT) and MuRF knockout (KO) mice submitted or not to the denervation procedure in the muscle (n = 3). D) Heatmap of atrophy and Rev-erbα pathways in the gastrocnemius of Wild-type (WT) and MuRF knockout (KO) mice that receive PBS or dexamethasone (Dexa) (n = 3).

gastrocnemius of humans. The authors verified a biphasic response following exercise, with an immediate increase postexercise, followed by a drop expression 1 h postexercise, and an addition to peak expression between 4 and 12 h postexercise [40].

The lower *Il-10* mRNA levels in the WTEx group do not corroborate the literature data. This result can be due to the half-life of mRNA being shorter than the analogous protein, which implies that transcriptional answers to an exercise stimulus are observable quickly [41]. The present

study should have analyzed the serum and protein content of IL-10.

Regarding the action of exercise on the atrophy pathway, in the present study, an increase in the *Trim63* (commonly known as *Murf1*) was observed in the IL-10 KO-Ex group, but without alteration of the *Fbxo32* gene (widely known as Atrogin-1), and the MuRF1 and Atrogin-1 proteins in the experimental groups. MuRF1 and Atrogin-1 play important roles in muscle atrophy and ubiquitin-mediated proteolysis [42]. Hinkley *et al.* [35], after a 20 km session on the cycle ergometer with 10 young adults, verified a significant increase in *Murf1* levels to the resting values in the *vastus lateralis* muscle. Still, chronically there was a decrease in its expression [35]. In another investigation using short-term endurance and resistance exercises [43] in healthy young adults, there was an increase in mRNA levels of *Murf1* and *Atrogin-1* after one endurance session in the *vastus lateralis* muscle. Another study analyzed the MuRF1 protein content immediately, 3, 6, and 12 h after an acute treadmill session [44]. Compared to the control group, MuRF1 protein content decreased immediately and after 3 h of exercise in the gastrocnemius muscle but increased 6 and 12 h after exercise [44].

Louis *et al.* [40] analyzed the time course of *Murf1* and *Atrogin-1* and visualized that *Murf1* mRNA increased 1, 2, and 4 h postexercise, respectively. *Atrogin-1* mRNA increased at 1, 2, and 4 h postexercise but with a lower increase when compared to *Murf1*. In the present study, the IL-10 KO-Ex showed higher levels of *Trim63* after 2 h postexercise, corroborating the literature findings [40]. Still, even after exercise, the WT group did not present any modulation, which makes us speculate that the presence of IL-10 protects against muscle atrophy and ubiquitin-mediated proteolysis after physical activity. While IL-10 primary role involves suppressing the production of pro-inflammatory cytokines, it can also induce macrophages to the M2c phenotype. These M2c macrophages share typical M2 markers, such as IL-4Rα, arginase-1, CD206 and CD163 [45]. CD163 + M2c macrophages are the predominant macrophage population during the regenerative stage of mdx muscular dystrophy, implying their potential influence on muscle regeneration [45], which could explain the higher levels of *Trim63* after exercise. We can speculate that after acute exercise, pro-inflammatory cytokines are released, and once the mice do not have the IL-10 to suppress the pro-inflammatory cytokines and induce the muscle regeneration by M2c phenotype, the mice did not reduce the number of injured fibers increasing the muscle atrophy gene.

In addition to verifying the genes and proteins associated with the atrophy pathway, it is important to note that the current experiment has a limitation. Specifically, the sectional area of the muscle fibers between the WT and IL-10 KO group was not assessed, which prevents us from determining whether the absence of IL-10 leads to a decrease in muscular mass, and injured fibers.

In the present study, there was no difference between the groups on the expression of the *Nr1d1* gene and the Rev-erbα protein, showing that this pathway was not directly affected by the absence of IL-10, even after the physical exercise intervention. Corroborating the present study, Rovina *et al.* [46] did not verify the modulation of *Nr1d1* mRNA levels in the gastrocnemius immediately after an acute running exercise (60 min at 60 % of maximum power). Using the same exercise protocol, da Rocha *et al.* [47] verified a decrease of *Nr1d1* mRNA levels in the gastrocnemius, but 12 h after the exercise. For the protein content, da Rocha *et al.* [47] verified a reduction in Rev-erbα in the gastrocnemius immediately after the acute running exercise. Pinto *et al.* [48] demonstrated a decrease of *Nr1d1* mRNA levels and Rev-erbα content in the gastrocnemius 1 and 3 h after 90 min of running exercise at 10° treadmill inclination. The difference between the studies can be due to the treadmill inclination and the time course of *Nr1d1* once this molecule oscillates during the day.

Using the data from GSE198266, the genes of *Nr1d1*, *Fbxo32* (*Atrogin-1*), and *Trim63* (*Murf1*) were higher for the young exercise group in the gastrocnemius and soleus muscle. Independently of exercise, mice submitted to an atrophy condition had lower *Nr1d1* levels. These public results reinforce the data by Mayeuf-Louchart *et al.* [28], showing that Rev-erbα counteracts atrophy. Future studies are needed to understand the relationship between Rev-erbα and its modulations in the different muscle fibers, as well as its relationship with IL-10.

Despite the non-modulation of *Nr1d1* and Rev-erbα, the *Bmal1* and *Clock* genes were lower in the IL-10 KO-Ex group, and BMAL1 protein levels were lower for the IL-10 group. While Bmal1 regulates circadian rhythm, adipogenesis, maintenance, and muscle function (43), Clock has structural and functional roles in the cell, both important for the muscular system [49] and sensitive to aerobic exercise. The Clock/Bmal1 complex performs Rev-erbα transcription, which represses the Bmal-1 transcription [49]. Pastore *et al.* [50] used mutant mice for the *Clock* and verified no modulation of the protein levels of CLOCK and BMAL1 after voluntary exercise in the wheel for eight weeks [50].

Circadian rhythms demonstrate a strong association with both immunity and metabolism [51]. Nevertheless, the extent and way the inherent circadian clock governs the immune response of cells remain uncertain. It was demonstrated, that in macrophages, Bmal1 controls inflammation by regulating the gene expression of *Nrf2,* which plays an important role in the innate immune system [52]. The deficiency of Bmal1 in macrophages derived from bone marrow results in augmented expression of IL-1β. Future studies should verify if Bmal1 and IL-10 are correlated via *Nrf2* in the skeletal muscle [52,53].

No studies have verified in skeletal muscle the Rev-erbα pathway in knockout animals to IL-10 after physical exercise. Other studies have demonstrated the same genes and proteins but in different conditions, models, and tissues. In a study with young adults (24.5 ± 0.8 years), Tanaka *et al.* [54] found that after a session on the cycle ergometer for 1 h at 60 % of maximum oxygen uptake, no difference was verified in the levels of the *Clock* gene in human leukocytes. Still, there was an increase in mRNA levels of *Bmal1* [54]. Investigating 26 obese and sedentary individuals (~66 years) who had prediabetes, Erickson *et al.* [55] found that 12 weeks of aerobic exercise on the treadmill significantly increased *Bmal1* gene expression levels in the *vastus lateralis* muscle. Despite this, there were no significant differences between pre and post-intervention for the *Clock* gene or on the BMAL1 and CLOCK proteins [55].

These divergent results may be due to differences in the population (humans, mutant mice, mice with or without IL-10), the intervention (acute or chronic exercise), the tissues used as samples, and the tissue collection time [56]. The lower levels of *Bmal1* and *Clock* genes observed in the IL-10 KO-Ex group suggest that the absence of this cytokine might play a crucial role in modulating these genes, particularly in physical exercise. However, further investigations involving different collection times and muscle tissues are necessary to fully comprehend the relationship between these molecular pathways.

To the best of our knowledge, this is the first investigation showing the relationship between Rev-erbα molecular pathways and modulation of atrophy genes in the absence of IL-10. Thus, it is concluded that IL-10 seems to be a determinant in the *Bmal1* and *Trim63* variation after acute exercise, with no modulation at baseline. In other words, the lack of IL-10 has negative implications for the mechanism that governs the circadian rhythm and loss of muscle mass, following a stimulus like physical activity. The current study, despite its limitations in sample size and failure to analyze the sectional area of muscle fibers, highlights the necessity for future investigations to incorporate this methodology in various skeletal muscles, particularly in a larger cohort of IL-10 knockout animals. Moreover, these future studies should also encompass different time points for data collection and explore the influence of these pathways on muscle response following a strength exercise protocol, given the robust association between IL-10 and muscle strength.

CRediT authorship contribution statement

Gustavo Eduardo da Mata: Formal analysis, Investigation, Writing – original draft, Visualization. **Rafael Bricola:** Investigation, Visualization. **Danielle Naves Ribeiro:** Investigation, Visualization. **Fernando M. Simabuco:** Resources, Writing – review & editing, Visualization.

José R. Pauli: Resources, Writing – review & editing, Visualization. **Ellen C. de Freitas:** Resources, Writing – review & editing, Visualization. **Eduardo R. Ropelle:** Resources, Writing – review & editing, Visualization. **Adelino S.R. da Silva:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Ana P. Pinto:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

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Data availability

Data will be made available on request.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT and Scispace to improve language and readability. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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